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journal homepage: www.elsevier.com/locate/procbioExploring the use of biosurfactants from *Bacillus subtilis* in bionanotechnology: A potential dispersing agent for carbon nanotube ecotoxicological studies

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ABSTRACT

In this work, we evaluate the efficiency of biosurfactants produced by *Bacillus subtilis* LSFM-05 for the dispersion of acid-treated multi-walled carbon nanotubes (CNT-LQES₁) and the effect of dispersion on toxicity testing with *Daphnia similis*. Carbon nanotubes are very hydrophobic materials and they readily agglomerate in mineral water. As a result, in order to determine their toxicity it is critical to evaluate methods to disperse these nanomaterials in a biologically compatible manner. The biosurfactant used in this work, termed BioS, which is a mixture of the lipopeptides (surfactin and fengycin), was found to be non-toxic to *D. similis* in an acute toxicity test (48 h) and it was an excellent dispersing agent for CNT-LQES₁ in reconstituted mineral water. Monitoring in real-time using the nanoparticle tracking analysis (NTA) showed that the colloidal stability of the CNT-LQES₁ suspension dispersed with BioS was highly stable. These findings are encouraging for the application of biosurfactants as nontoxic dispersion agents in the emerging fields of bionanotechnology and nanotoxicology.

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1. Introduction

Bionanotechnology is a new area that has emerged from the convergence of biotechnology and nanotechnology. A primary challenge in this emergent area is understanding the interaction of nanoscale materials with biological systems, with the aim of producing functionalized nanomaterials with applications in health, agriculture and the environment.

Carbon nanotubes (CNTs) have attracted great scientific interest due to their unique physico-chemical and biological properties.

These exciting properties have inspired the application of CNTs and their engineered derivatives in the development of electronic devices, nanocomposites, drug delivery systems and the remediation of persistent organic contaminants [34,35]. Regarding their environmentally related applications, CNTs are promising materials for sensing and removal of important pollutants such as dyes, pesticides, heavy metals and polycyclic aromatic hydrocarbons (PAHs) present in water, air and soil [26]. Recently, we have demonstrated that acid-treated multi-walled carbon nanotubes, termed CNT-LQES₁, are able to interact with mutagenic pollutants [32].

The CNTs industry is growing rapidly, with prospects of a wide range of CNT based products with the potential to affect human and environmental health. The global industrial production of CNTs has been estimated to be 100–1000 tons/year [25]. Consequently, it is inevitable that CNTs will be released into the environment during their life cycle. The release of nanomaterials into the environment will occur mainly through the decomposition and recycling of nanocomposite materials and in the wastewater discharge of CNT

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manufacturing industries, if not appropriately treated. In addition, nanomaterials present in fabrics, paints and cosmetics can reach water streams through laundering processes [13].

However, despite the potential applications of CNTs, there currently is no consensus on the safety aspects of CNTs due to the limited and non-standardized ecotoxicological protocols and assays so far employed for their hazard assessment [5,23,31]. CNTs are allotropes of carbon comprised a rolled-up graphene sheet in the form of a cylindrical structure. They can be divided into two different groups according to the number of layers: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). Both nanomaterials are very hydrophobic and have a strong tendency to form agglomerates in the aqueous media used in most ecotoxicity testing protocols recommended by regulatory agencies [e.g., Organization for Economic Cooperation and Development (OECD), The United States Environmental Protection Agency (USEPA), and Brazilian Association for Technical Standards (ABNT)]. As a result, the colloidal stability of dispersed CNTs has been found to be one of the most important considerations for the assessment of ecotoxicity of CNTs [29,30].

Dispersion of CNTs in mineral water has been achieved using synthetic polymers and surfactants, proteins, phospholipids and humic substances [4,28]. Besides having emulsifying activity, the dispersant agents should possess low toxicity toward the model organism utilized in the assay. Therefore, the use of low toxicity dispersing agents in ecotoxicological studies is critical to guarantee that the results obtained are directly associated with the CNTs themselves and have been minimally influenced by the toxicological effects of the dispersing agent or the solvent used in the evaluation. Thus, it is of great importance to identify dispersing agents that can interact with CNTs and improve their dispersability and stability in water without toxic effects [9,11].

Biosurfactants are natural surface-active compounds derived from bacteria, fungi, animals and plants. They are amphiphilic molecules consisting of a hydrophobic and a hydrophilic moiety. Typically, the non-polar domain is the hydrocarbon component of a fatty acid and the polar group can be constituted of sugars (glycolipids), peptides (lipopeptides), or polysaccharides (polymeric surfactants). *Bacillus subtilis* is a well studied producer of a variety of structurally diverse cyclic lipopeptide biosurfactants, the most studied of these being surfactin, fengycin and iturin, which are known for their ability to reduce surface and interfacial tension and also to form very stable oil/water emulsions. Generally, when compared with their synthetic counterparts biosurfactants offer several advantages such as low toxicity, high biodegradability and effectiveness at extreme conditions of pH, temperature and salinity [2]. Because biosurfactants are recognized as “green” and ecologically safe products, some reports have pointed out applications to these biomolecules in nanotechnology [12,20].

Recently, our group purified and characterized the biosurfactants (surfactin and fengycin) produced by *B. subtilis* LFSM-05 [6,7]. These resulting biosurfactants mixtures (designated BioS) were characterized using nuclear magnetic resonance spectroscopy (NMR), Fourier transform infrared (FT-IR) spectroscopy and high-resolution mass spectrometry (ESI-FTMS) techniques. Due to their surface-active properties, we chose to explore the use of these biosurfactants as biocompatible agents to disperse and assess the ecotoxicological properties of carbon nanotubes.

The aim of this work was evaluate the potential use of biosurfactants produced by *B. subtilis* LFSM-05, termed BioS, as emulsifying agents in the dispersion process of the acid-treated multi-walled carbon nanotubes (CNT-LQES₁). *Daphnia similis* was used as the test organism to assess the acute toxicity of carbon nanotubes suspended in reconstituted mineral water, with or without dispersion with a non-toxic concentration of BioS. Furthermore, the colloidal stability of CNT-LQES₁ suspensions was also monitored

by nanoparticle tracking analysis (NTA) which is an innovative and powerful technique to study the colloidal stability of nanomaterials in liquid suspensions during toxicity testing [8,10].

2. Materials and methods

Industrial grade multi-walled carbon nanotubes (MWCNT, *Tube 100*) were commercially obtained from CNT Co. Ltd. (Incheon, Korea). *B. subtilis* LFSM-05 was obtained from the biological collection of the Laboratory of Systematic and Microbial Physiology (LSFM) at the Faculty of Food Engineering, University of Campinas (UNICAMP), São Paulo, Brazil. The raw glycerol used as the carbon source for growth of *B. subtilis* LFSM-05 was donated by Granol Ltda (Anápolis, Goiás, Brazil). Reconstituted mineral water used in toxicity testing (pH 7.2, conductivity 160 $\mu\text{S cm}^{-1}$ and hardness 44 mg/L of CaCO_3) was prepared by adding $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (1.2×10^{-4} mol/L), KCl (3.0×10^{-5} mol/L), NaHCO_3 (6.0×10^{-4} mol/L) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4.0×10^{-4} mol/L) to mineral water from the Próspera natural fountain (Serra Negra, São Paulo, Brazil).

2.1. Preparation of the acid-treated multiwall carbon nanotubes

The industrial grade MWCNT *Tube 100* (1.0 g) was submitted to acid treatment under reflux using HNO_3 (7.0 mol/L) for 24 h at 140 °C followed by similar treatment with HCl (5.0 mol/L) for 6 h at 110 °C. After the oxidation process the MWCNTs were vacuum-filtered through a 0.2 μm PTFE membrane and extensively washed with deionized water until the filtrate reached a neutral pH value. The acid-treated MWCNTs were dried in a vacuum system for 24 h and this sample was named CNT-LQES₁. CNT-LQES₁ was characterized using several physicochemical techniques in an integrated way as earlier described by [32]. In summary, the nanotube diameter distribution varied from 10 to 40 nm and nanotube length was found to be less than 10 μm . Surface area (BET method, ASAP 2010 Micromeritics instrument) and surface charge (ζ -potential, nano-ZS Malvern instruments) of CNT-LQES₁ were $260 \pm 10 \text{ m}^2/\text{g}$ and $-27 \pm 8 \text{ mV}$, respectively. The I_D/I_G ratio (structural defect index) of CNT-LQES₁ was 1.64 ± 0.3 and was measured using Raman spectroscopy (TS-150 WITHEC spectrometer). The final content of metallic residue (iron oxide) in the CNT-LQES₁ sample was less than 1.0% (Analytical microbalance, AD-6 Perkin-Elmer). Transmission electronic microscopy (TEM), scanning electronic microscopy (SEM) and thermogravimetric analysis (TGA) of CNT-LQES₁ are presented in Supplementary material (Fig. S1).

Supplementary figure related to this article found, in the online version, at doi:10.1016/j.procbio.2014.04.006.

2.2. Preparation and isolation of biosurfactants produced by *B. subtilis* LFSM-05

The protocol used for biosurfactant production was previously reported by [6,7]. Briefly, the microorganism was grown in a culture medium containing raw glycerol as carbon source (5%, v/v). The fermentation process was performed in a 15-l bench-top fermentor (Bioflo 3000, New Brunswick Scientific) at 32 °C with stirring at 250 rpm and aeration rate of 0.5 vvm for 72 h, without addition of chemical anti-foam. The crude biosurfactant extract was recovered from the foam overflow formed during the fermentation process. After allowing the foam to collapse, the remained cells were removed by centrifugation (18,000 $\times g$ for 10 min at 4 °C) and the biosurfactants in the supernatant were precipitated by adding concentrated hydrochloric acid. The supernatant was maintained at 4 °C overnight and the culture medium was centrifuged, re-suspended in deionized water and lyophilized to produce the crude extract. Subsequently, the crude extract (1.0 g) was suspended in

100 mL of reconstituted mineral water (the same solution used in acute ecotoxicity assay with *D. similis*). This suspension was subjected to magnetic stirring for 2 h at room temperature to solubilize the biosurfactants. After this process, the suspension was centrifuged at 3000 rpm for 10 min. The soluble fraction (supernatant) was collected and designated BioS. The BioS suspension consisted of a mixture of surfactin and fengycin lipopeptides produced by *B. subtilis* LSFM-05 as reported by [6,7].

2.3. Dispersion method and stability studies

A stock-solution was prepared by dispersing 100 mg of CNT-LQES₁ in deionized water (100 mL). This dispersion was sonicated for 1 h using an ultrasound bath (Cole-Parmer 8891). The sonicated CNT-LQES₁ stock-solution was then diluted to a range of concentrations (from 1.0 to 90 mg/L). These dilutions were performed using reconstituted mineral water with and without BioS at 0.25 g/L. The CNT-LQES₁ dilutions were sonicated for 30 min in order to ensure good contact between BioS and the carbon nanotubes. The suspension stability of the dispersions was quantitatively monitored using a spectrophotometer. The quantity of carbon nanotubes in the suspension was determined by measuring the optical density at 500 nm [17]. The colloidal stability and agglomeration phenomena of these suspensions were also monitored using the Nanoparticle Tracking Analysis (NTA) technique (LM10 equipment, software version 2.0 – NanoSight Ltd, Amesbury, England). After introduction into the NanoSight chamber, all samples were maintained in standby mode for 30 s to achieve equilibrium. Videos were recorded over 10 s with camera parameters (focus, shutter and gain) visually adjusted and the software parameters (brightness, gain and blur) were also adjusted for each sample for optimal visualization. The temperature used in the test was measured with assistance of an external thermometric sensor.

2.4. Acute ecotoxicity assay

Acute ecotoxicity tests were performed using *D. similis* as test organism. Different concentrations of the acid-treated MWCNTs (CNT-LQES₁) and biosurfactants (BioS) were tested separately. CNT-LQES₁ dispersed in BioS solution (0.25 g/L) was also tested. Tests were carried out in four replicates. For each replicate, five organisms of *D. similis* (6–24 h old) were exposed to the samples for 48 h at a temperature of 18–22 °C in a 10 mL plastic tube, without light. After an exposure period of 48 h, the immobilized *D. similis* were counted. The test was considered valid when the immobilization rate was less than 10% in the negative control group. The results were statistically analyzed using the Trimmed Spearman–Kärber method for estimating the median immobilization concentration. Optical microscopy was employed to visualize *D. similis* after the treatments with BioS, CNT-LQES₁ and CNT-LQES₁ dispersed in BioS.

3. Results and discussion

Pristine carbon nanotubes are extremely hydrophobic and insoluble materials, therefore the oxidation of CNTs with nitric acid is a common method to functionalize the CNTs surface, improving the dispersion of CNTs in water. These oxidative treatments are able to introduce structural defects, thus creating functional groups containing oxygen (e.g., hydroxyl, carbonyl, ketones and carboxyl) along the CNTs surface. These oxygenated groups provide electrostatic stabilization when the oxidized CNTs are suspended in water. However, the density and location of these oxygenated groups as well as oxidation debris generation are strongly dependent on the oxidation conditions employed such as acid concentration, temperature and time [1].

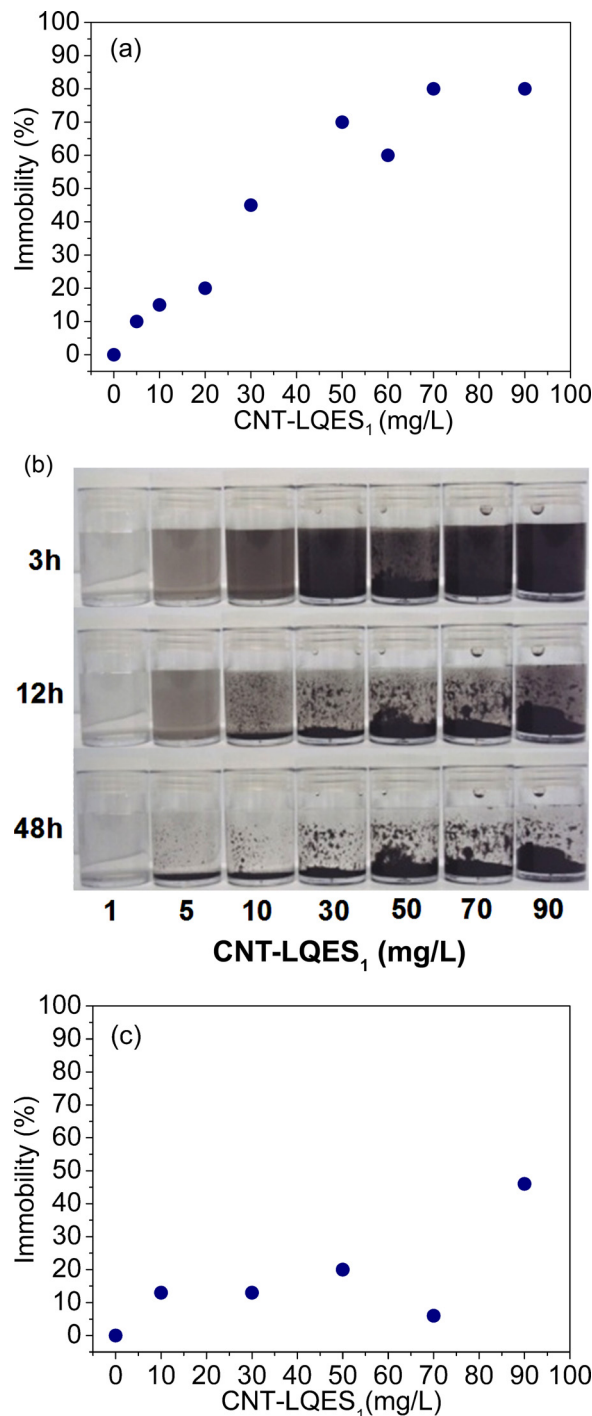


Fig. 1. (a) Immobility of *Daphnia similis* after 48 h of exposure to CNT-LQES₁ dispersions in reconstituted mineral water (from 1.0 to 90 mg/L). (b) Photos of CNT-LQES₁ dispersions in the reconstituted mineral water used in the acute ecotoxicity assay over time (from 3 to 48 h). (c) Immobility of *Daphnia similis* after 48 h of exposure to CNT-LQES₁ dispersions where the neonates were introduced 2 h after nanotube dispersion.

The acid-treated CNT-LQES₁ used in this paper can be well dispersed in deionized water for a long time (up to 48 h) (Supplementary material, Fig. S2), but not in reconstituted mineral water as shown in Fig. 1b. This fact can be explained by the presence of high concentration of electrolytes in reconstituted water used (i.g. 44 mg/L of CaCO₃). The lack of colloidal stability in aqueous solution containing electrolytes has also been reported for other kinds of nitric acid oxidized MWCNTs [21]. As a consequence, various

studies have been performed to identify biocompatible molecules able to disperse CNTs to facilitate the assessment of their toxicity and allow biotechnological applications [9,11]. The acute ecotoxicity of acid-treated CNT-LQES₁ was evaluated using an immobility assay with *D. similis* after 48 h of exposure to several concentrations of CNT-LQES₁ (from 1.0 to 90 mg/L) (Fig. 1a). The EC₅₀ of immobilization was 36.4 mg/L. However, after 48 h in contact with reconstituted mineral water, CNT-LQES₁ became highly agglomerated and settled to the flask bottom, demonstrating that the carbon nanotubes did not form a stable suspension in the reconstituted mineral water used in the acute ecotoxicity assays (Fig. 1b).

Supplementary figure related to this article found, in the online version, at [doi:10.1016/j.procbio.2014.04.006](https://doi.org/10.1016/j.procbio.2014.04.006).

We expected that the bioavailability of CNT-LQES₁ would be reduced as a result of agglomeration. Therefore, to evaluate if the nanotube agglomeration settling events had a significant impact on the acute toxicity of the carbon nanotubes toward *D. similis* the neonates were introduced into the bioassay flasks 2 h after initial CNT-LQES₁ dispersion. After 48 h of exposure to the CNT-LQES₁, the number of immobilized organisms was counted (Fig. 1c). As a consequence of the preagglomeration and settling, acute toxicity, in terms of immobility, was dramatically reduced as compared to the results shown in Fig. 1a. Moreover, the fact that an agglomeration period of only 2 h resulted in such significant reduction in toxicity indicates that reduction in bioavailability occurs well before significant visual observation of agglomerated material occurs. The reduction in toxicity likely was a result of agglomeration process forming larger particle that prevented the ingestion of carbon nanotubes by *D. similis*, thus mitigating the toxicity of larger CNT-LQES₁ particles. These results strongly indicate that the nanotube agglomeration phenomenon in reconstituted mineral water is a critically important interfering factor to be considered when assessing carbon nanotube ecotoxicity.

CNTs are a heterogeneous class of nanomaterials and their toxicity assessment can be also influenced by their specific physicochemical properties (e.g., size distribution, surface area, functional groups, structural defects and purity) [14]. As a result, it is very difficult to compare the CNTs ecotoxicity data to *Daphnia* organisms (in terms of EC₅₀ or LC₅₀ values) in the current literature [16,23].

Li et al. [15] demonstrated that surfactin, a biosurfactant isolated from *B. subtilis*, interacts with SWCNTs making them dispersible in deionized water. Ximenes et al. [33] have also demonstrated that several biopolymers, including the surfactin, can interact with pristine SWCNTs, allowing for the formation of stable suspensions in deionized water. However, this is the first report in the literature about the use of surfactin and fengycin molecules for dispersing MWCNT combined with evaluation of the ecotoxicity of the resulting suspension.

To ensure that the BioS was not influencing the toxicity testing of CNT-LQES₁, the acute ecotoxicity of BioS was evaluated using *D. similis*. The tested sample showed a very low acute toxicity value toward this organism (EC₅₀ = 0.4 g/L) (Fig. 2) in comparison to the synthetic surfactant dodecyl sodium sulfate (SDS) with reported EC₅₀ between 0.009 and 0.030 g/L [19]. To the best of our knowledge, this is the first time that ecotoxicity of biosurfactants produced by *B. subtilis* was evaluated using *D. similis*. Lima et al. [18] also evaluated the acute toxicity of biosurfactants produced by *Bacillus* sp. LBBMA 111A and *B. subtilis* LBBMA 155 on the bioluminescent bacterium in a *Vibrio fischeri* bioluminescent test. The EC₂₀ values obtained demonstrate a low toxicity of these biosurfactants in comparison to the synthetic surfactant SDS.

To evaluate the toxicity of CNT-LQES₁, a fixed concentration (0.25 g/L) of BioS was selected because it did not present toxic effects toward *D. similis* (Fig. 2). The dispersability tests were performed using reconstituted mineral water described in Section 2.3. In Fig. 3a it can be clearly observed that the biosurfactant was able

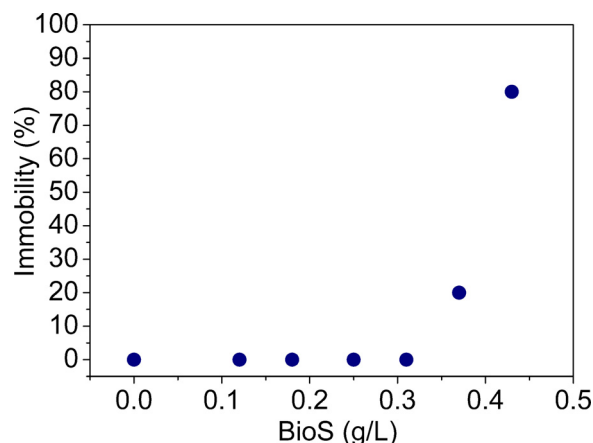


Fig. 2. Immobility of *Daphnia similis* after 48 h exposure to the biosurfactants produced by *Bacillus subtilis* LSFM-05 (BioS).

to stabilize the CNT-LQES₁ in reconstituted mineral water over a period of 48 h of exposure. However, the optical density measurements demonstrated that a concentration of 30 mg/L is the maximum concentration of CNT-LQES₁ that can be stabilized using

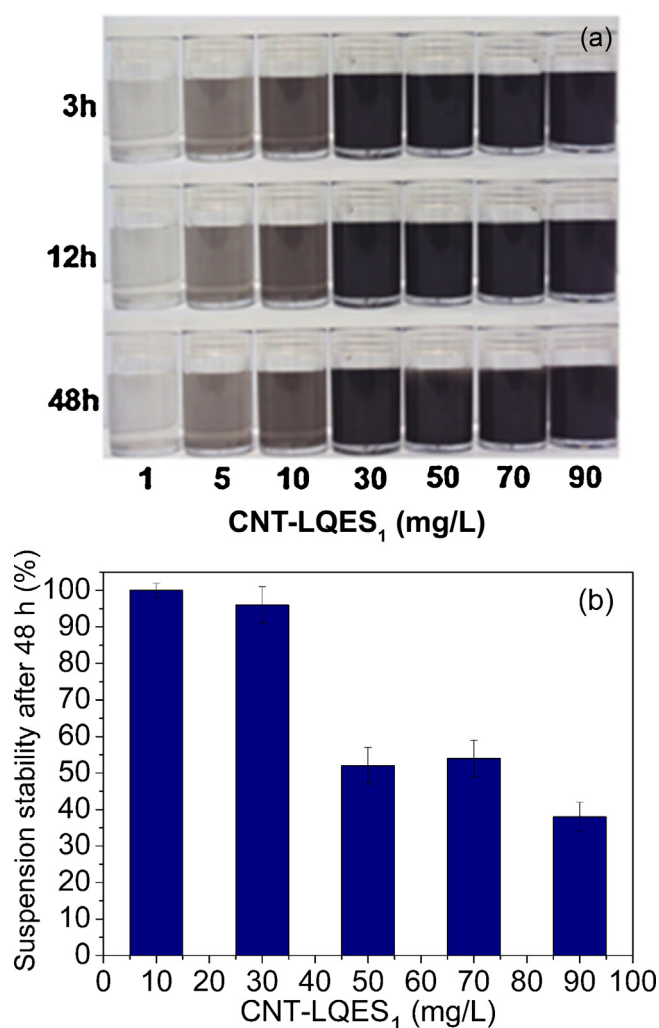


Fig. 3. (a) Photographs of CNT-LQES₁ suspensions (from 1.0 to 90 mg/L) after dispersion with BioS in the reconstituted mineral water used in the ecotoxicity assay over time (from 3 to 48 h). (b) Suspension stability of CNT-LQES₁ in reconstituted mineral water after their dispersion with BioS.

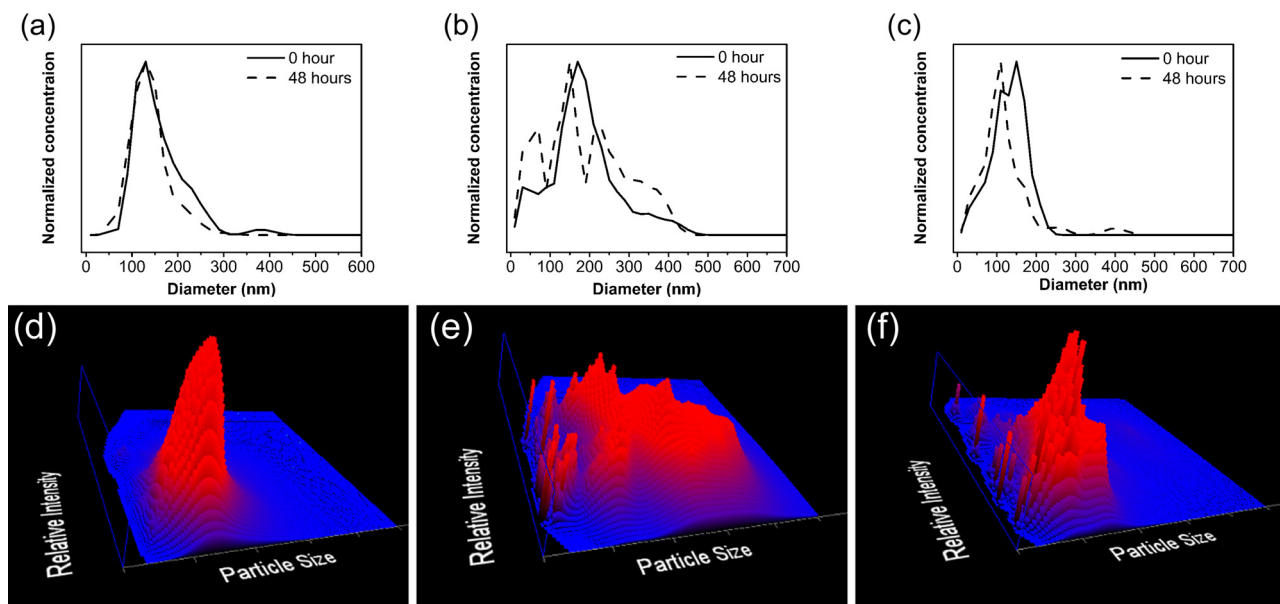


Fig. 4. Real-time monitoring (0 h and 48 h) of CNT-LQES₁ suspensions (5.0 mg/L) in (a) deionized water, (b) reconstituted mineral water and (c) after dispersion with BioS in reconstituted mineral water measured using nanoparticle tracking analysis (NTA). Particle size distribution of CNT-LQES₁ suspensions after 48 h, in (d) deionized water, (e) reconstituted mineral water and (f) dispersed with BioS in reconstituted mineral water.

a non-toxic BioS concentration of 0.25 g/L. At this concentration, over 90% suspension stability was obtained (Fig. 3b). This result indicates that BioS is superior in forming stable suspensions of carbon nanotubes, in comparison to typical synthetic surfactants (e.g., CTAB, NaDBS, SDS, F127, F68 and TX100), which have been shown able to disperse CNTs in aqueous solutions at concentrations varying from 1.0 to 20 mg/L [3]. However, the type of carbon nanotubes studied as well as the type of water used in toxicity tests can influence the dispersion performance. Therefore, the surfactant concentration needed to stabilize the CNTs can also vary depending on the nanomaterial surface properties as well as the type of organism used in the tests. For example, *Daphnia magna* requires harder water than *D. similis*, which could also play a role in the dispersion performance of any surfactant.

New approaches and techniques are needed to improve the knowledge about the colloidal stability of CNTs and their impacts on toxicity. Recently, Schwyzer et al. [30] reported on long-term colloidal stability of 10 different types of CNTs in the absence and presence of humic acid and calcium using the NTA technique to monitor the agglomeration events of CNTs in a buffered suspension (MOPS, pH 7.0). Due to the utility of this technique we also used NTA for characterization of the agglomeration of dispersed CNT-LQES₁. NTA technique is an innovative method that can be used to analyze small particles (nano or micro scale) in liquid suspensions, thus allowing the determination of their size distribution profile. The NTA technique is unique because it is able to calculate the particle size on a particle-by-particle basis, where each particle is simultaneously visualized and tracked by a dedicated particle tracking image analysis program. Consequently, it is possible to detect real-time events of nanomaterials agglomeration using the NTA technique [8,10].

The CNT-LQES₁ suspensions were monitored by NTA after their dispersion in deionized water and in reconstituted mineral water with and without BioS. Particle size increase was clearly observed as function of time (from 0 h to 48 h) in reconstituted mineral water due to agglomeration of nanotubes. However, these events were not observed when CNT-LQES₁ was dispersed in deionized water or in reconstituted mineral water containing BioS (Fig. 4). According to the NTA-particle size distribution the average size of the

particles was about 130 nm both in deionized water or reconstituted water containing BioS (Fig. 4a and c). However, after dispersion in reconstituted water without BioS for 48 h (Fig. 4b) three independent particle diameter peaks developed, which is consistent with the CNTs agglomeration process. Fig. 4d–f shows 3D representations of the hydrodynamic diameters of the particles present in the measured samples. The number of particles present in the media containing only BioS (without CNT-LQES₁) was checked and it was found to be negligible (data not shown).

The mechanisms involved in the stabilization of CNTs in water systems using synthetic and natural surfactants are not fully understood [24]. However, a possible mechanism could be the chemical interaction of the hydrophobic moiety of surfactin and fengycin molecules with the aromatic groups on the nanotube surface. The adsorption of BioS molecules on CNTs surface could reduce the π - π stacking interactions among carbon nanotubes, decreasing nanotube bundle formation and increasing their stability in reconstituted water. Some authors have suggested that the surfactin is capable of forming cylindrical micelles with a β -sheet

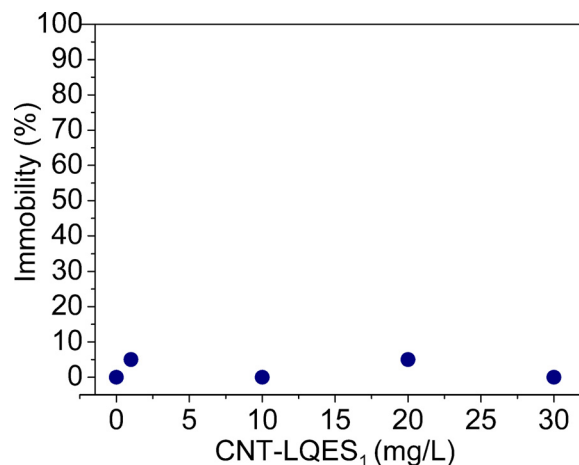


Fig. 5. Immobility of *Daphnia similis* after 48 h exposure to CNT-LQES₁ dispersed with BioS at a non-toxic concentration.

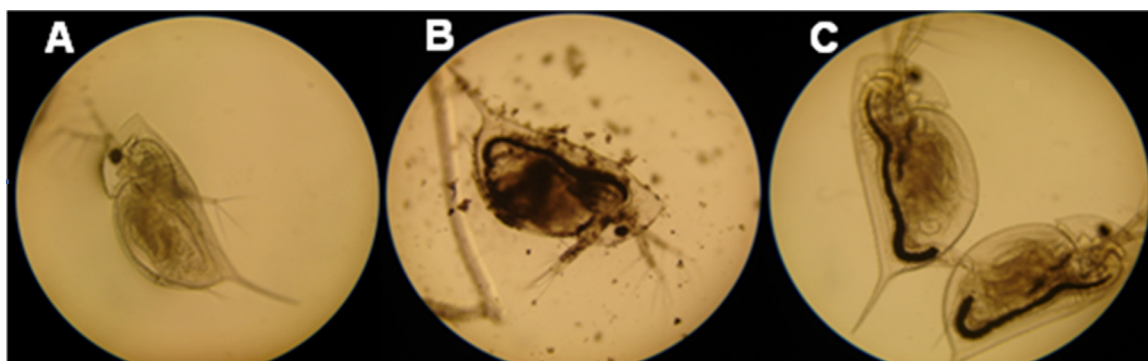


Fig. 6. Optical microscopy images of *Daphnia similis* after 48 h exposure to (A) BioS, (B) CNT-LQES₁ (5.0 mg/L) and (C) CNT-LQES₁ (5.0 mg/L) dispersed with BioS.

configuration, and this structure could be responsible for water dispersion of pristine SWCNTs [15,33].

CNT-LQES₁ stabilized with BioS did not show toxicity to *D. similis* in a concentration range from 1.0 to 30 mg/L (Fig. 5). This result could be explained by the reduction of hydrophobic properties of the CNT-LQES₁ caused by the BioS coating effect on the nanotube surface. Hydrophobic substances such as CNTs can easily adhere to *Daphnia* biological material [36]. Fig. 6 shows micrographs of *D. similis* in the presence of BioS, CNT-LQES₁ and CNT-LQES₁ dispersed with BioS. In the case of the acute test performed with CNT-LQES₁ alone it is possible to observe the carbon nanotube agglomerates interacting with the *Daphnia* carapace or body surface (Fig. 6b). Large amounts of dark material were also found in the gut tract of *D. similis* after CNT-LQES₁ exposure but not in the control (Fig. 6a). After the dispersion of CNT-LQES₁ with BioS the agglomerates were not observed (Fig. 6c). Although the BioS dispersed nanotubes did not cause immobility to *D. similis* until 30 mg/L, the nanomaterial was observed inside and localized in the gut of *D. similis*, as shown in Fig. 6c. When these organisms were moved to a clean solution (without the presence of carbon nanotubes), the organisms quickly (less than 1 min) released the nanomaterial present inside the gut, without the observation of any further immobility effect in the organisms that remained in that solution.

Roberts et al. [27] have studied the toxicity of lipid-coated SWCNTs to *D. magna*. The authors reported that the organisms were able to ingest the nanotubes through their normal feeding behavior. In addition, *D. magna* were able to modify the solubility of the nanotube, likely through digestion of the lipid coating (lysophosphatidylcholine), which they consumed as a food source. In contrast, [22] reported the uptake and depuration of SWCNTs by *D. magna* showing that these organisms were unable to excrete the nanotubes accumulated on their guts after 24 h of depuration on artificial fresh water or filtered Lake Kantiolampi water. Further, Gao et al. [9] have demonstrated that the process of dispersing SWCNTs with gum arabic, a non-toxic natural surfactant, can mitigate the acute toxicity of nanotubes against the green algae *Pseudokirchneriella subcapitata* and the crustacean *Ceriodaphnia dubia*, both aquatic model organisms commonly used in ecotoxicological assays. These results demonstrate that the biomodification of surface chemistry of CNTs is an important phenomenon that should be considered in future ecotoxicity studies of carbon nanotubes.

Finally, it is interesting to highlight that the production of the biosurfactant used in this work was based on raw glycerol derived from biodiesel industry. This represents an opportunity to use an inexpensive and abundant agroindustrial waste for the low cost production of a valuable biosurfactant for use in bionanotechnology and nanotoxicology. Additionally, hybrid biosurfactant CNT systems may find environmental applications in the remediation of

pollutants considering that individually both materials have been used in several types of environmental applications.

4. Conclusions

A mixture of biosurfactants produced by *B. subtilis* LSFM-05 (surfactin and fengycin) using raw glycerol as substrate, termed BioS, was shown to efficiently disperse acid-treated multiwalled carbon nanotubes (designated CNT-LQES₁) in reconstituted mineral water commonly used in standardized aquatic ecotoxicity tests. The CNT-LQES₁ dispersed with BioS did not show acute toxicity against *D. similis* until a concentration of 30 mg/L. From an environmental perspective, these are important results regarding the ecotoxicity effects of biosurfactant-coated carbon nanotubes. Our findings suggest new opportunities for exploring the use of microbial biosurfactants in nanotechnology as greener and safer modifiers of carbon nanotubes and others carbon nanostructures.

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